ELSEVIER

Biochimica et Biophysica Acta 1593 (2002) 45–55

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Phosphorylation of the cell cycle inhibitor p21$^{Cip1/WAF1}$ by Pim-1 kinase

Zeping Wang[a], Nandini Bhattacharya[a], Philip F. Mixter[a], Wenyi Wei[b], John Sedivy[b], Nancy S. Magnuson[a,*]

[a] *School of Molecular Biosciences and the Cancer Prevention and Research Center, Washington State University, P.O. Box 644234, Pullman, WA 99164-4234, USA*

[b] *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA*

Received 16 August 2002; accepted 23 September 2002

Abstract

The serine/threonine kinase, Pim-1, appears to be involved in regulating proliferation, differentiation and cell survival of lymphoid and myeloid cells. In this study, we have found that amino acid residues 140–147 (RKRRQTSM) at the C-terminal end of p21$^{Cip1/WAF1}$, a cyclin-dependent kinase (CDK) inhibitor, constitute an ideal phosphorylation consensus sequence for Pim-1. We demonstrate that Pim-1 efficiently phosphorylates this peptide sequence as well as the p21 protein in vitro. We also demonstrate by pull-down assay and by immunoprecipitation that Pim-1 associates with p21. During phorbol ester-induced differentiation of U937 cells, both Pim-1 and p21 expression levels increase with Pim-1 levels increasing in both the nucleus and cytoplasm while p21 remains primarily cytoplasmic. Co-transfection of wild type p21 with wild type Pim-1 results in cytoplasmic localization of p21 while co-transfection of wild type p21 with kinase dead Pim-1 results in nuclear localization of p21. Consistent with the results from the phosphoamino acid assay, Pim-1 phosphorylates transfected p21 only on Thr^{145} in p21-deficient human fibroblasts and this phosphorylation event results in the cytoplasmic localization of p21. These findings demonstrate that Pim-1 associates with and phosphorylates p21 in vivo, which influences the subcellular localization of p21.

Keywords: Protein kinase; Oncogene; Phosphorylation; Cell cycle inhibitor p21$^{Cip1/WAF1}$

1. Introduction

Cell cycle progression results from the coordinated expression and interaction of positive regulatory proteins, including cyclins and their associated cyclin-dependent kinases (CDK), and negative regulatory proteins, CDK inhibitors [1]. The first identified and most extensively studied CDK inhibitor is p21$^{Cip1/WAF1}$ (hereafter referred to as p21). p21 is known to directly inhibit kinase activities of a wide range of cyclin/CDK complexes, resulting in a general arrest of the cell cycle [2,3]. It also binds to the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ, which leads to inhibition of DNA replication directly without affecting DNA repair [4,5]. Besides functioning in cell cycle arrest and DNA synthesis control, p21 has also been shown to be involved in many processes including differentiation, stress response, apoptosis, development and tumorigenesis (reviewed in Refs. [6–8]). Consistent with its numerous cellular functions, p21 has been found to participate in a number of specific protein–protein interactions. In addition to associating with cyclin CDKs and PCNA [2–4], p21 has also been reported to bind to transcription factors (c-Myc [9], E2F [10], C/EBP-α [11], STAT3 [12]), proteins involved in apoptosis (procaspase 3 [13], stress associated protein kinase (SAPK) [14], apoptosis signal regulating kinase (ASK1) [15]) and other proteins (SET [16], calmodulin [17], HPV-16 E7 [18], GADD45 [19], TOK-1 [20], and protein kinase CK 2 [21]).

The proto-oncogene Pim-1 was originally identified as a preferential integration site of Moloney murine leukemia virus, which induces T-lymphomas in mice [22]. Oncogenicity of Pim-1 has been well documented in both transgenic and retroviral models [23,24]. By itself, Pim-1 has low oncogenic potential but cooperates strongly with c-Myc, L-

* Corresponding author. Tel.: +1-509-335-0966; fax: +1-509-335-1907.
E-mail address: magnuson@mail.wsu.edu (N.S. Magnuson).

0167-4889/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0167-4889(02)00347-6

Myc, N-Myc, Bcl-2 and Gfi-1 in T-cell lymphomagenesis [25–28]. Besides functioning in lymphomagenesis, Pim-1 has also been shown to be involved in the IL-3 signal transduction pathway, in cell cycle regulation and proliferation, in apoptosis and cell survival (reviewed in Ref. [29]) [30].

Pim-1 is a highly conserved serine/threonine kinase and its preferential phosphorylation consensus sequence was demonstrated to be $(Arg/Lys)_4$-X-Ser/Thr-X′, where X′ is likely to be neither a basic nor a large hydrophobic residue [31,32] Currently, eight proteins have been reported to be phosphorylated by Pim-1. These are p100 (a transcriptional co-activator of c-Myb) [33], cdc25A phosphatase (a cell cycle phosphatase) [34], HP1 (heterochromatin protein 1, a transcriptional repressor) [35], PAP1 (Pim-1 associated protein 1, whose function is unknown) [36], PTP-U2S (a tyrosine phosphatase) [37], NFATc1 (a transcription factor) [38], TFAF2/SNX6 [39] and nuclear mitotic apparatus protein (NuMA) [40]. Because Pim-1 is involved in multiple cellular activities, it is very likely that Pim-1 has more substrates than presently identified.

Because p21 has a potential Pim-1 phosphorylation site located in the C-terminal end of p21, we were interested to determine if p21 might also be a substrate for Pim-1. In this study, we have identified p21 as a Pim-1-associating protein in vivo and have demonstrated that p21 protein is phosphorylated by Pim-1 specifically on Thr^{145}. We also demonstrate by co-transfection of p21 null cells that wild type Pim-1 causes a translocation of p21 to the cytoplasm whereas p21 remains nuclear in the presence of the kinase dead Pim-1.

2. Materials and methods

2.1. Cell culture and differentiation induction

Human monoblastoid leukemia U937 cells (ATCC CRL-1593.2) were grown in RPMI 1640 media (Gibco, Grand Island, NY) plus 10% FBS. The p21 null human fibroblast cells were cultured as previously described [41]. Phorbol ester (PMA), 10 ng/ml culture was used to induce U937 cell differentiation during a 48 h period.

2.2. Plasmids and transfections

The cDNA for human p21 (provided by Dr. Bert Vogelstein, The Johns Hopkins University School of Medicine and The Howard Hughes Medical Institute) was subcloned into the expression vector pBK/CMV (Stratagene, La Jolla, CA). To replace the Thr^{145} with Asp or Ala, a PCR-based site-directed mutagenesis kit (Stratagene) was used. The primer for T145D was CCAGGACAGCATGACAGATTTCTACCACTCCAAAC and the primer for T145A was CAAGCTTCCATGACAGATTTCTACCACTCCCAAAC. Transfection of p21 null fibroblasts was carried out using lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.3. In vitro kinase assay

Kemptide peptide LRRASLG was purchased from Sigma. The p21 peptide RKRRQTSM, the p21 reverse peptide MST QRRKR, the modified p21 peptides RKRRQTAM and RKR RQASM, the cdc25A peptide KRRKSM and the histone H1 peptide KRRASGP were synthesized on campus. Kinase assays were performed as previously described [42]. Phosphorylation was quantitated on a Packard 1900 TR liquid scintillation analyzer.

2.4. Protein extraction and Western blot analysis

At different times after the addition of PMA, U937 cells were lysed by suspension in RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 14 mM 2-mercaptoethanol, and 2 mM EDTA) followed by gentle sonication. The proteins (40 μg) were separated on 12% SDS polyacrylamide gel by electrophoresis and were transferred onto PVDF membrane (Fisher Scientific, Pittsburgh, PA). For Pim-1 analysis, the membranes were then blocked in PBS/Tween (PBS with 5% non-fat powered milk and 0.05% Tween 20) at room temperature for 2 h. The blot was then incubated with affinity-purified rabbit anti-Pim-1 at 1:100 in PBS/Tween for 1 h. The membranes were washed with PBS/Tween for 5, 10 and 15 min and incubated with horseradish peroxidase conjugated goat anti-rabbit antibody diluted 1:20,000 (Pierce, Rockford, IL) for 1 h at room temperature. After repeating the washing steps, the signal was detected by chemiluminescence using the Pierce Super Signal West Pico System (Pierce). Membranes were stripped for reprobing by incubating in stripping buffer (2% SDS, 62.4 mM Tris–HCl pH 6.8, 100 mM 2-mercaptoethanol) for 30 min at 50 °C. The membranes were then reblocked and probed for p21 or actin. Anti-p21 antibody (C-19) and anti-actin antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Nuclear and cytoplasmic fractionation was carried out as previously described [43]. During differentiation at selected time points, 400 μl cytoplasmic extract and 400 μl nuclear extract were made from 10^7 U937 cells. Equal volumes of protein (30 μl) from cytoplasmic or nuclear fractions were separated on 12% SDS-PAGE and subjected to Western blot with anti-Pim-1 antibody or anti-p21 antibody.

2.5. Phosphorylation of immunoprecipitated p21 and phosphoamino acid analysis

Cellular extracts were prepared as described above. Proteins (500 μg) were gently rotated with 1 μg of anti-p21 antibody and 15 μl of protein G agarose (Boehringer Mannheim, Germany) at 4 °C for 1 h. The immunocomplex was washed five times with RIPA buffer and then used in a kinase assay as previously described [42]. In the peptide competition assay, 1 μl of 10 mM p21 peptide or p21 reverse peptide was included in the reaction. After 30 min, the reaction

mixture was boiled in Laemmli sample buffer and phosphorylated proteins were separated on a 10% SDS polyacrylamide gel. The resulting gel was visualized by autoradiography.

For phosphoamino acid analysis, phosphorylated p21 and Pim-1 were separated by SDS-PAGE and transferred to a PVDF membrane. The portion containing phosphorylated p21 was excised after autoradiography and the membrane was washed five times with deionized water. One-dimensional and two-dimensional thin layer chromatography (TLC) analyses were performed according to the method described previously [44,45].

2.6. In vitro "pull-down" assay

GST-Pim-1 and GST were generated as previously described [31]. Two micrograms of the purified GST-Pim-1 or GST was first applied to Glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden) in RIPA buffer. After extensive washing of the glutathione sepharose 4B/GST-Pim-1 or GST complex, 200 μg of U937 cell lysates were added and incubated with the complex for 1 h at 4 °C. With the peptide competition, increasing amounts of 10 mM p21 peptide (1, 5 or 10 μl) or 10 μl of 10 mM p21 reverse peptide was added to the reaction mixture, respectively. The complexes were extensively washed with RIPA lysis buffer and then suspended in Laemmli buffer, boiled for 5 min, and separated on 12% PAGE. p21 and GST-Pim-1 proteins were detected by Western blot analysis as described above.

2.7. Immunoprecipitation assay

U937 cell lysates (500 μg) were gently mixed with 1 μg anti-Pim-1 antibody and protein G-agarose (Boehringer Mannheim) for 1 h at 4 °C and washed with RIPA buffer five times. The bound proteins were separated by 12% SDS-PAGE, blotted onto a PVDF membrane and reacted with anti-Pim-1 antibody or with C-19 anti-p21 antibody.

2.8. Indirect immunofluorescence

Following two washes in 1 × PBS, the U937 cells were fixed in 80% methanol for 20 min. Nonspecific antibody binding was blocked by incubation with 3% bovine serum albumin (BSA) in 1 × PBS for 1 h at 4 °C followed by a 1 h incubation with specific antibodies diluted 1:50 in 1.5% BSA. A polyclonal rabbit Pim-1 peptide antibody (StressGen, Vancouver, Canada), raised against the N-terminal peptide sequence of residues 1-37 of Pim-1, was used to localize Pim-1 protein. To localize p21 in the two cell types, a monoclonal mouse IgG, WAF-1(Ab-1) was used (Oncogene, Cambridge, MA). The unbound antibodies were removed by three 5-min washes in 1.5% BSA. Goat anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC; Cappel Scientific Division, Malvern, PA) was diluted 1:50 in 1.5% BSA and applied to the coverslips for 1 h as a secondary antibody to Pim-1. Donkey anti-mouse IgG antibody conjugated to Cy™5 (Jackson Laboratories, Bar Harber, ME) was used in a 1:50 dilution in 1.5% BSA to localize p21. Unbound conjugated antibody was removed with three washes in 1.5% BSA and one wash in 1 × PBS. Coverslips were mounted on microscope slides (Fisher Scientific) using the mounting medium Vectashield (Vector Laboratories, Burlingame, CA) that contained propidium iodide. Detection and imaging was achieved using a Bio-Rad 1024 laser confocal microscope (Bio-Rad Laboratories, Hercules, CA). The specific wavelengths for fluorescent visualization were 522 nm for FITC, 598 nm for propidium iodide and 750 nm for Cy™5 and the three images were merged by computational manipulation. Pre-immune serum was used as a negative control for Pim-1 and antibody specificity was verified by a competition assay (data not shown). For staining p21 in p21 null fibroblasts, a mouse monoclonal antibody against human p21 (1:100, Santa Cruz) was used. The second antibody was goat anti-mouse IgG conjugated to Oregon green (1:400, Molecular Probes).

3. Results

3.1. Pim-1 kinase phosphorylates a peptide derived from the C-terminal end of p21 protein

The C-terminal end of p21 protein contains the sequence RKRRQTSM (amino acid residues 140–147) that is a preferred phosphorylation sequence for Pim-1 [2,3,32,42]. By using the GST fusion protein with either wild type or kinase dead (mutated ATP binding site) Pim-1, we found that only GST-Pim-1 (wild type), but not the GST-Pim-1 (kinase dead) or GST alone, could phosphorylate the C-terminal peptide of p21 (Fig. 1A). However, the GST-Pim-1 (wild type) did not phosphorylate the p21 reverse peptide (MSTQRRKR) (Fig. 1B). We further evaluated the phosphorylation of p21 peptide by comparing its phosphorylation with that of peptides previously used to evaluate Pim-1 phosphorylation activity. As shown in Fig. 1B, p21 peptide is highly phosphorylated by Pim-1 compared to the other substrates previously shown to be phosphorylated by Pim-1, which includes H1 peptide, Kemptide and cdc25A peptide. These results show that the p21 peptide is efficiently phosphorylated by the wild type Pim-1, and p21 is a better substrate than other peptide substrates reported previously.

3.2. Preferential phosphorylation of residue Thr^{145} of p21 protein

The C-terminal peptide of p21 (RKRRQTSM) contains two potential Pim-1 phosphorylation sites next to one another, Thr^{145} and Ser^{146}. Thr^{145} has been shown to be phosphorylated by PKA and Akt while Ser^{146} has been shown to be phosphorylated by PKC [46,47]. Phosphor-

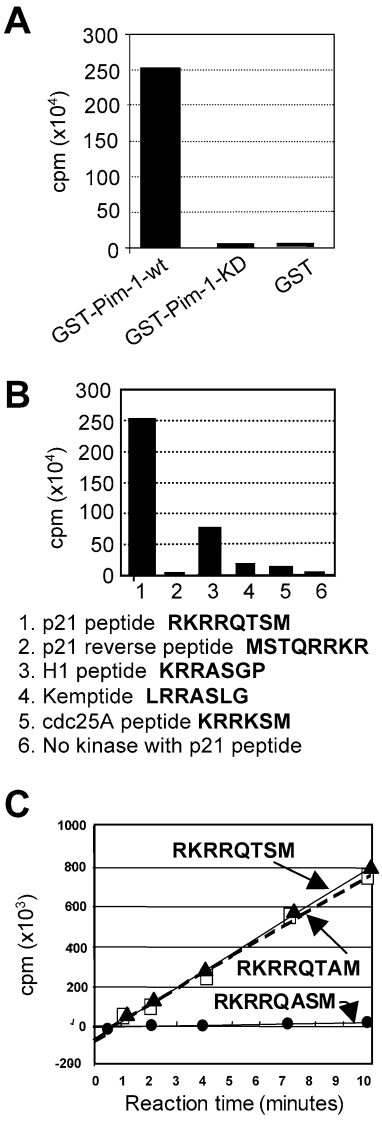

Fig. 1. Phosphorylation of p21 peptide by Pim-1 kinase. (A) Phosphorylation of p21 peptide by GST-Pim-1-wt (wild type) but not by GST-Pim-1-KD (kinase dead) or by GST. ^{32}P incorporation is expressed as counts per minute (cpm). (B) Comparison of the phosphorylation of different peptides by Pim-1 kinase. (C) The kinetics of the phosphorylation of p21 peptide (▲) and the two modified p21 peptides (RKRRQTAM, □; RKRRQASM, ●) by GST-Pim-1 (wild type). Each point is the mean of triplicate samples and lines represent the calculated trend for the data.

ylation of either residues would appear to be important in regulating p21 associating with PCNA and also possibly its nuclear localization based on the nuclear localization signal at residues 140–153, which includes both Thr^{145} and Ser^{146} [8,48]. Therefore, it was of interest to determine if one or the other of the residues would be preferentially phosphorylated by Pim-1 kinase. Two modified p21 peptides (RKRRQTAM and RKRRQASM) with either the Thr^{145} or Ser^{146} changed to alanine were synthesized. Phosphorylation of the p21 peptide and the modified p21 peptides were conducted to evaluate the efficiency of phosphorylation by Pim-1 kinase at residues Thr^{145} and Ser^{146}. The phosphorylation of the three different p21 peptides showed that peptides with Thr^{145} were readily phosphorylated as compared to the peptide with only Ser^{146} (Fig. 1C). In addition, the amount of ^{32}P incorporation is almost identical for both the p21 peptide (RKRRQTSM) and the modified p21 peptide (RKRRQ TAM), while the amount of ^{32}P incorporation in p21 peptide (RKRRQASM) was extremely low. These results suggested that phosphorylation of p21 peptide by Pim-1 occurred only on Thr^{145}.

3.3. Phosphorylation of p21 protein by Pim-1 in vitro

In order to determine if Pim-1 kinase would phosphorylate p21 protein in vitro, p21 protein was immunoprecipitated from PMA-treated U937 cells and subjected to the kinase assay (Fig. 2). We found that the p21 protein was phosphorylated by GST-Pim-1 but not by kinase dead GST-Pim-1 or by GST alone. When the p21 peptide was added as a competitive inhibitor in the kinase reaction, the phosphorylation of the p21 protein was greatly inhibited. However, when the same amount of p21 ‘‘reverse’’ peptide was included in the

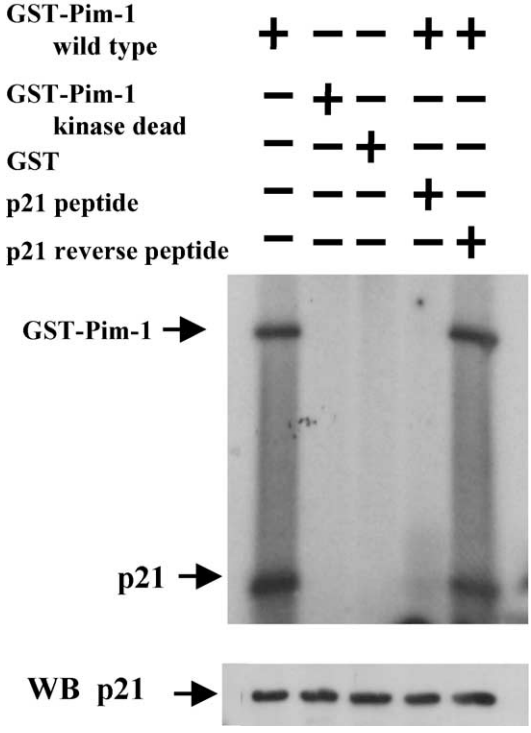

Fig. 2. In vitro phosphorylation of p21 protein by Pim-1 kinase. p21 protein was immunoprecipitated from PMA-treated U937 cell lysates and used as substrate in the Pim-1 kinase assay. GST-Pim-1 (wild type), GST-Pim-1 (kinase dead), and GST were expressed in *Escherichia coli*, purified for use in the kinase assay. Phosphorylated proteins were separated by 12% SDS-PAGE and visualized by autoradiography. p21 peptide and p21 reverse peptide were put into the kinase assay as competitors with the p21 protein. The lower panel shows Western blot (WB) for p21 used in the phosphorylation reactions. Data are representative of three independent experiments.

reaction, there was no effect on the phosphorylation of p21 protein. This result shows that Pim-1 kinase specifically phosphorylates p21 protein in vitro.

3.4. Pim-1 phosphorylates p21 on Thr^{145}

To provide further information about the specific site being phosphorylated by Pim-1 in the p21 protein, a phosphoamino acid assay was carried out. p21 protein was immunoprecipitated from U937 cells and subjected to the kinase assay with Pim-1 kinase. Phosphorylated p21 proteins were subjected to phosphoamino acid analysis. Both one- and two-dimensional TLC were used to determine which residues were being phosphorylated by Pim-1 [44,45]. We found that p21 was only phosphorylated on threonines providing further support for our hypothesis that the preferential residue being phosphorylated by Pim-1 is Thr^{145} (Fig. 3).

3.5. Binding of p21 protein to Pim-1 in "pull-down" assays

In order to test whether p21 protein associates with Pim-1, cell lysates from PMA-treated U937 cells were prepared and incubated with GST-Pim-1 attached to glutathione-sepharose 4B beads. After the beads were washed, they were boiled in sample buffer and the liberated proteins were separated by SDS-PAGE and Western blotted with anti-p21 antibody. We found that p21 protein specifically associated with the Pim-1 on the beads (Fig. 4, lane 1). When the p21 peptide was added to the lysate–GST–Pim-1 bead mixture, the peptide was able to compete for the binding to the Pim-1. With increasing amounts of added p21 peptide, the amount of associated p21 protein with Pim-1 beads decreased (Fig. 4, lanes 2–4). However, the p21 reverse peptide was not capable of competing with the p21 protein for binding to GST-Pim-1 (Fig. 4, lane 5). No p21 protein was observed with beads attached with only GST (data not shown). These results demonstrate that Pim-1 specifically associates with p21 protein in vitro.

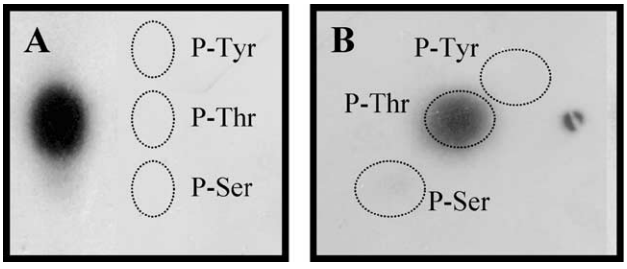

Fig. 3. Phosphoamino acid analysis of p21. p21 was immunoprecipitated from U937 cell lysates, phosphorylated by Pim-1, separated by SDS-PAGE and transferred to PVDF membrane. Phosphorylated p21 was excised and hydrolyzed with HCl. The amino acid composition was determined by either (A) one-dimensional TLC or (B) two-dimensional TLC. The dotted circles indicate the position of the control phosphoamino acids.

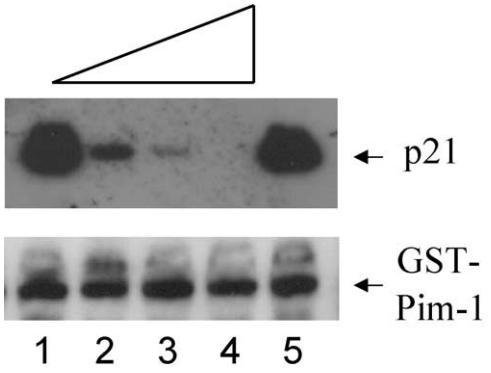

Fig. 4. Association of p21 protein with Pim-1 kinase in a "pull-down" assay. p21 protein was isolated from PMA-treated U937 cell lysates on GST-Pim-1 bound to glutathione sepharose 4B beads and subjected to Western blot analysis with anti-p21 antibody. Lane 1 shows p21 protein bound by the GST-Pim-1 beads. Lanes 2, 3 and 4 demonstrate successful competition of p21 protein from binding to GST-Pim-1 beads by increasing amount of p21 peptide. Lane 5 shows that the p21 reverse peptide is not able to compete with p21 protein for binding to the GST-Pim-1 beads. The lower panel shows the equal amount of GST-Pim-1 by Western blotting with anti-Pim-1 antibody. This result is representative of three independent experiments.

3.6. Binding of p21 protein to Pim-1 in immunoprecipitation assays

Interaction between p21 protein and Pim-1 was also evaluated by immunoprecipitation assay. Cell lysates were prepared from PMA-treated U937 cells and precipitated with either anti-Pim-1 antibody or non-specific IgG. The precipitates were then subjected to Western blot analysis using either anti-p21 antibody or anti-Pim-1 antibody (Fig. 5). p21 protein was detected in whole lysates and only the precipitate with the anti-Pim-1 antibody but not in the precipitate with IgG. These results indicate that p21 protein associates with Pim-1 in vivo in U937 cells.

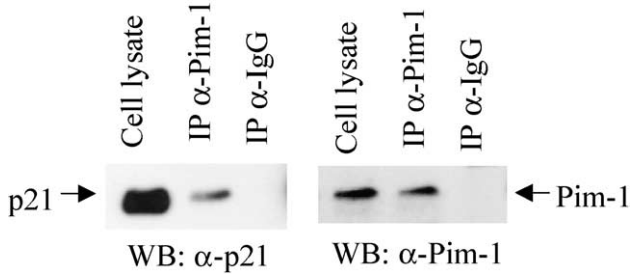

Fig. 5. Association of p21 protein with Pim-1 as determined by immunoprecipitation (IP). Cell lysates from PMA-treated U937 cells (48 h) were immunoprecipitated with anti-Pim-1 antibody or non-specific IgG. Precipitates were separated by 12% SDS-PAGE and subjected to Western blot (WB) analysis with either anti-Pim-1 antibody or anti-p21 antibody. The positions of the p21 and Pim-1 proteins are indicated by arrows. Similar results were obtained in three other independent experiments.

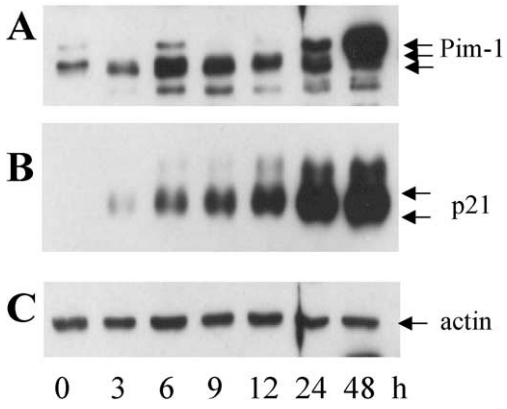

Fig. 6. Kinetics of p21 and Pim-1 expression during U937 cell differentiation. Cell lysates from U937 cells were prepared at different time points following PMA-induced differentiation. The proteins were separated by 12% SDS-PAGE and subjected to Western blot analysis sequentially with either anti-Pim-1, anti-p21 or anti-actin antibodies. Similar results were obtained in two other independent experiments.

3.7. Kinetics of p21 and Pim-1 expression during U937 cell differentiation

Because p21 was found to bind to Pim-1 and to be phosphorylated by Pim-1, it was of interest to examine the expression pattern of p21 and Pim-1 proteins simultaneously during U937 cell differentiation. After inducing differentiation with PMA, both Pim-1 and p21 levels increased gradually (Fig. 6). At 48 h post-stimulation, a time point when monocytic differentiation was well underway (Fig. 8), Pim-1 and p21 proteins were highly expressed. As a control, we also demonstrated that there was no change in the expression level of actin during U937 cell differentiation.

3.8. Cellular localization of p21 and Pim-1 during the course of U937 cell differentiation

Previous studies have shown that p21 is found primarily in the nucleus of myeloid cells induced to differentiate with vitamin D3, with translocation to the cytoplasm later during the course of differentiation [15].

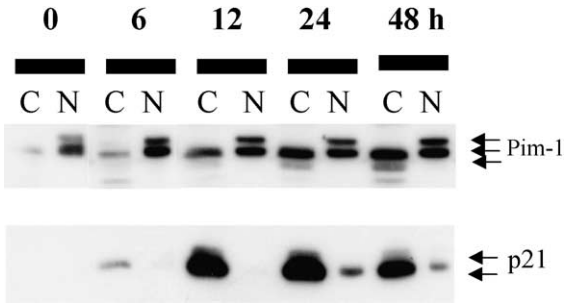

Fig. 7. Subcellular localization of p21 and Pim-1 during differentiation of U937 cells. Nuclear (N) and cytoplasmic (C) fractionation of U937 cells was prepared at different time points during PMA-induced differentiation. Proteins from various fractions were separated by 12% SDS-PAGE and subjected to Western blot analysis with both anti-p21 and anti-Pim-1 antibodies. These findings were confirmed in two other independent experiments.

In order to determine if there was a correlation between the localization of Pim-1 with that of p21 during PMA-induced differentiation, the cytoplasm and nucleus of U937 cells induced to differentiate with PMA were analyzed separately using Western blot. As shown in Fig. 7, the localization of Pim-1 proteins is seen primarily in the nucleus early during differentiation (6–12 h) and then appears to increase in both the cytoplasm and the nucleus later in differentiation (24–48 h). Most of p21 is found in the cytoplasm starting as early as 6 h with levels increasing steadily to the 48 h time point. However, the appearance of p21 is just barely detectable in the nucleus at 6–12 h. Increases are seen primarily in the cytoplasm from 12 to 48 h. These samples were also evaluated by confocal microscopy confirming the Western blot results. As can be seen in Fig. 8, the cellular morphology indicates that the U937 cells are undergoing differentiation at 24 h. This is clearly demonstrated in column 3 by the change in nuclear shape from round (0 h) to the classic kidney bean shape (48 h). Before treatment with PMA, Pim-1 protein can be seen in both the cytoplasm and nucleus (column 2). However, after 24 h exposure to PMA, there is an increase of Pim-1 level in the nucleus. By confocal microscopy, the level of Pim-1 in the

Fig. 8. (top) Distribution of Pim-1 and p21 proteins during U937 cell differentiation. U937 cells were induced to differentiate with 10 ng/ml of PMA. The cells were stained respectively at 6, 24 and 48 h after the addition of PMA to study the distribution of Pim-1 and p21 by confocal laser scanning microscopy. Cells not induced with PMA are controls (0 h) as shown in the top row. Column 1 (blue) represents cells stained for p21 (Cy™5). In Column 2, FITC (green) label indicates Pim-1 distribution while in Column 3 the nuclei have been stained with propidium iodide (red). Column 4 results from the computational merging of Pim-1 (green) and the nucleus (red) shown as a yellow color. Column 5 represents the merged images of Pim-1 (green) and p21 (blue) as shown by a light blue color. The computational merging of all three labels is in column 6 showing the distribution of both proteins, Pim-1 and p21, within the nucleus and the cytoplasm.

Fig. 9. (bottom) Pim-1 alters the cellular localization of p21. (A) Wild type (Wt) Pim-1 or kinase dead (KD) Pim-1 and p21 were co-transfected into p21 null human fibroblasts at a ratio 10:1. The localization of p21 was visualized by immunostaining with anti-p21 antibody. When wild type Pim-1 was co-transfected with p21, p21 was localized in the cytoplasm (top row). However, when kinase dead Pim-1 was co-transfected with p21, p21 was localized in the nucleus (bottom row). (B) When p21 T145A was transfected into p21 null human fibroblasts, the p21 was localized in the nucleus. When p21 T145D was transfected into p21 null human fibroblasts, the p21 was localized in the cytoplasm. (C) When p21 T145D was co-transfected with wild type Pim-1 or kinase dead Pim-1, p21 was localized in the cytoplasm. When p21 T145A was co-transfected with wild type Pim-1 or kinase dead Pim-1, p21 was localized in the nucleus.

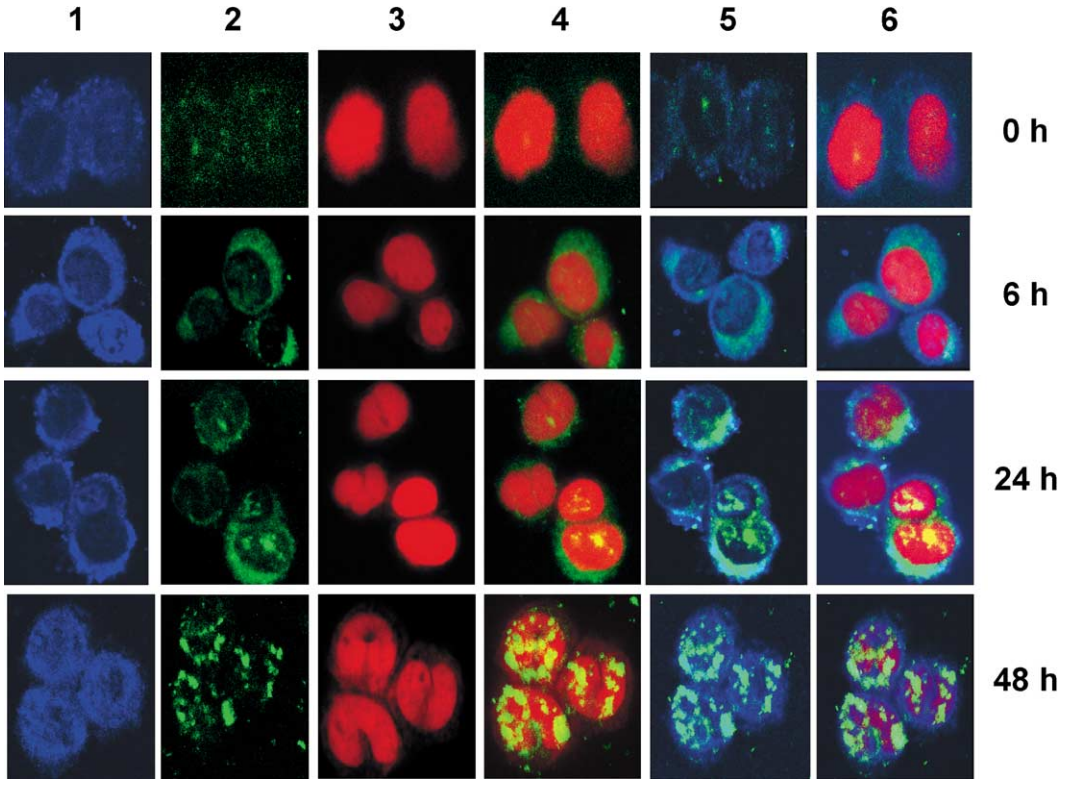
1
2
3
4
5
6
0 h
6 h
24 h
48 h

Fig. 8.

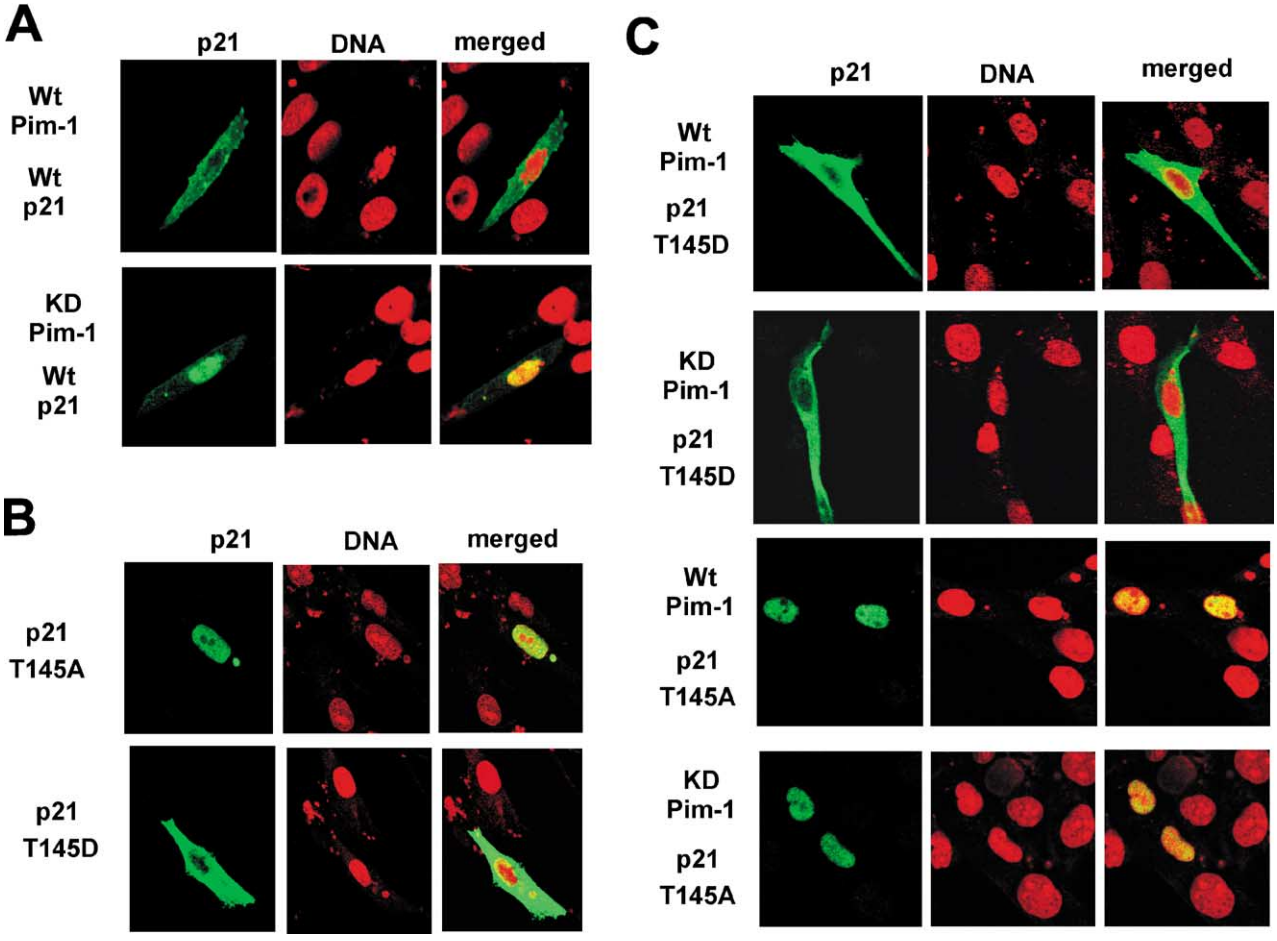
A
p21
DNA
merged
Wt Pim-1
Wt p21
KD Pim-1
Wt p21
B
p21
DNA
merged
p21 T145A
p21 T145D
C
p21
DNA
merged
Wt Pim-1
p21 T145D
KD Pim-1
p21 T145D
Wt Pim-1
p21 T145A
KD Pim-1
p21 T145A

Fig. 9.

cytoplasm is not as evident as detected by Western blot (Fig. 7), and part of this observation may be due to the apparent aggregation of Pim-1 as detected by confocal microscopy at 48 h. In contrast, p21 appears to be predominantly in the cytoplasm before PMA treatment (column 1) and does not appear to change its overall location after 24 h exposure to PMA. The general distribution of Pim-1 and p21 protein is more evident when one compares the merged images, columns 5 and 6. Therefore, there is a difference in the induction of differentiation by Vitamin D3 and PMA, in that with PMA very little p21 is found in the nucleus at the early stage of differentiation. However, the similarity between these inducers is that p21 tends to be primarily cytoplasmic during differentiation.

3.9. Pim-1 induces cytoplasmic localization of p21

Our observations that p21 associates with and is phosphorylated by Pim-1 encouraged us to determine the biological function of this phosphorylation event. Therefore, p21 null fibroblasts were co-transfected with either wild type p21 and wild type Pim-1 or with wild type p21 and kinase dead Pim-1. The cellular localization of p21 in transfected cells was determined by immunostaining with anti-p21 antibody. As shown in Fig. 9A, the presence of the wild type Pim-1 resulted in the distribution of p21 primarily in the cytoplasm. However, with the kinase dead Pim-1, p21 was found almost exclusively in the nucleus. This result demonstrates that in vivo phosphorylation of p21 by Pim-1 results in the cytoplasmic localization of p21 in a manner similar to that seen for Akt [47].

3.10. Mutation of Thr^{145} of p21 to aspartic acid mimics phosphorylation and influences its subcellular localization

To further demonstrate the effect of phosphorylation of p21 on its cellular localization, we mutated Thr^{145} to either alanine (to eliminate the Pim-1 phosphorylation site) or to aspartic acid (to mimic the phosphorylated form of p21). By transfection of either p21 T145A or p21 T145D into p21 null fibroblasts, we were able to see that changing the charge on the Thr^{145} of p21 was enough to influence its cellular localization. As shown in Fig. 9B, when p21 T145A was transfected into cells, p21 was found to be almost exclusively nuclear. However, when p21 T145D was transfected into cells, p21 was found to be localized primarily in the cytoplasm.

3.11. Pim-1 phosphorylates p21 on Thr^{145} in vivo

To demonstrate that Pim-1 phosphorylation of p21 on Thr^{145} determines the cellular localization of p21, p21 T145D and p21 T145A were co-transfected with wild type Pim-1 or kinase dead Pim-1, respectively, into p21 null human fibroblasts. As shown in Fig. 9C, when p21 T145D was co-transfected with wild type Pim-1 or kinase dead Pim-1, the p21 was localized predominantly in the cytoplasm. However, when p21 T145A was co-transfected with either wild type Pim-1 or kinase dead Pim-1, the localization of p21 was found to be primarily in the nucleus. This result indicates that Pim-1 phosphorylation of p21 in vivo occurs only on residue Thr^{145}, and not on Ser^{146} because it was available for phosphorylation, and that this phosphorylation event leads to cytoplasmic localization of p21.

4. Discussion

In this report, we describe experiments demonstrating that Pim-1 directly binds to and phosphorylates the CDK inhibitor p21 at Thr^{145}. During PMA-induced differentiation of U937 cells, expression levels of Pim-1 kinase increase in both the cytoplasm and nucleus, while p21 protein increases primarily in the cytoplasm (Figs. 6 and 7). We observed that during the early stage of differentiation (0–12 h), Pim-1 kinase was localized primarily in nucleus, but p21 protein was found to be cytoplasmic and almost undetectable in the nucleus. This might be due to the nuclear p21 protein being phosphorylated by Pim-1 kinase and then translocated to the cytoplasm as was observed when Pim-1 and p21 were co-transfected into p21-deficient human fibroblasts (Fig. 9). However, at the later stage of U937 cell differentiation (24–48 h), a small amount of p21 was also detected in the nucleus although there were high levels of nuclear Pim-1 kinase. This might be explained by the fact that during induced differentiation of myeloid cells, that the serine/threonine phosphatases, PP1 and PP2A, are up-regulated [49–51]. It is likely that these phosphatases dephosphorylate p21 protein in the cytoplasm allowing p21 to translocate back into the nucleus. Therefore, the localization of p21 at any point during differentiation would be determined by the balance of phosphatase and kinase activities.

Phosphorylation of p21 by Pim-1 kinase has important implications for the potential regulation of p21 function. First, phosphorylation of p21 has been associated with the growth arrest and differentiation of PC12 nerve cells [52]. Secondly, recent studies show that phosphorylation of p21 within residues 140–147 (RKRRQTSM), which also is the preferred phosphorylation site of Pim-1, results in the inhibition of p21–PCNA complex formation [46]. When bound to PCNA, p21 is degraded more slowly compared with p21 binding to cyclin/CDK, which increases the rate of p21 degradation [53]. Thirdly, phosphorylation of p21 protein is also necessary for p21/procaspase 3 complex formation that hinders Fas-mediated cell death [54]. Finally, as mentioned in the introduction, p21 has more than 10 different binding partners. It is interesting that a single small molecule can form high-affinity complexes with such a large spectrum of different targets. However, it has been shown that p21 is a very flexible protein with little or no secondary

structure [55]. It is also possible that several kinases can phosphorylate p21 at different sites and thereby further modify its secondary structure to conform to the shape of the target molecules. Besides Pim-1, three protein kinases, PKC, PKA and Akt have been reported to phosphorylate p21 [46,47] and all may have a significant impact on p21 function in vivo.

These three kinases, PKC, PKA and Akt, are constitutively expressed but require some sort of post-translational modification for activity via an activation signal initiated at the surface of the cell. For example, PKC requires lipid second messengers (diacylgycerol) and phosphorylation (reviewed in Ref. [56]), PKA requires the second messenger, cAMP and A-kinase anchor proteins (reviewed in Ref. [57]) and Akt requires the lipid phosphatidylinositiol 3,4,5-triphosphate and multiple phosphorylations from an upstream kinase, PDK1 [58–60]. Therefore, one might expect that the levels of the active form of these kinases are not always present continuously or simultaneously under normal physiological conditions. Pim-1, on the other hand, is constitutively expressed at low levels but increased levels are rapidly induced in response to variety of cytokine, hormone, antigen or mitogen stimulation. Unlike PKC, PKA and Akt, Pim-1 is constitutively active ([32]; Magnuson, unpublished data). It is noteworthy that the consensus sequences that PKC, PKA, Akt and Pim-1 preferentially phosphorylate are somewhat different from each other but with potential overlap for similar "target" sequences (Table 1). In addition, the potential impact of these kinases on controlling the phosphorylation state of p21 would also depend on activating signals and cell type. In any case, it would be naïve to assume that only one kinase would be solely responsible for regulating the biological function of p21. More realistically, it is probably the combined activity of two or more of these kinases that when expressed and/or activated, facilitate regulation of p21 function.

Table 1
Comparison of the preferred phosphorylation consensus sequences of kinases shown to phosphorylate p21 at the site **RKRRQTSM**

Kinase	Preferred consensus sequence	Reference
PKC[a]	R/K-X-R/K-R/K-X-S/T-**F**-R/K-R/K	[61]
PKA	RXS, RRXS, KRXXS, RXXS	[57]
Akt	RXXRXX-S/T	[62,63]
Pim-1[b]	R/K-R/K-R-R/K-X-S/T-X, where X is an amino acid with a small side chain but neither basic nor acidic	[32,42]

[a] Optimal peptide sequences were determined using an oriented library of more than 2.5 billion peptide substrates for each of nine human PKC isozymes (α, βI, βII, γ, δ, ε, ν, μ and ζ). Although there were differences between the optimal substrates for each isozyme, all PKC isozymes preferentially phosphorylated peptides with hydrophobic amino acids at position +1 carboxyl-terminal of the phosphorylated S/T and with basic residues at −3.

[b] The preferred phosphorylation consensus sequence was determined by stepwise replacement of the amino acids in peptide substrate analogues based on the carboxyl-terminal segment of human ribosomal protein S6 (residues 229–249).

p21 has been shown to be involved in multiple functions. In addition to functioning in differentiation and apoptosis, p21 has also been shown to associate with development, tumor suppression, senescence, and regulation of cellular signal transduction [6–8]. Most of these cellular functions directly depend on the interaction of p21 with a number of associated proteins. Phosphorylation of p21 by Pim-1 regulates its subcellular distribution, which in turn allows binding to proteins which themselves have distinct subcellular locations, thus, potentially influencing numerous cellular functions attributed to p21. Therefore, this might explain in part why Pim-1 is involved in such diverse cellular functions (differentiation, proliferation and cell survival) because one of its substrates is also involved in multiple cellular functions.

In summary, our studies demonstrate that Pim-1 interacts with p21 (both in vitro and in vivo) and that Pim-1 can phosphorylate p21 in vivo and regulate its subcellular distribution. Our finding of p21 as a substrate for Pim-1 kinase provides a partial explanation for the multiple functions of Pim-1 kinase.

Acknowledgements

This work was supported in part by the National Institutes of Health Grants RO1 AG16694 and T32-AI07025 and by the Cancer Prevention and Research Center, Washington State University.

References

[1] C.J. Sherr, J.M. Roberts, CDK inhibitors: positive and negative regulators of G1-phase progression, Genes Dev. 13 (1999) 1501–1512.
[2] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, Cell 75 (1993) 805–816.
[3] Y. Xiong, G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, D. Beach, p21 is a universal inhibitor of cyclin kinases, Nature 366 (1993) 701–704.
[4] S. Waga, G.J. Hannon, D. Beach, B. Stillman, The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA, Nature 369 (1994) 574–578.
[5] M.K. Shivji, S.J. Grey, U.P. Strausfeld, R.D. Wood, J.J. Blow, Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair, Curr. Biol. 4 (1994) 1062–1068.
[6] K. Harada, G.R. Ogden, An overview of the cell cycle arrest protein, p21 (WAF1), Oral Oncol. 36 (2000) 3–7.
[7] J. Boulaire, A. Fotedar, R. Fotedar, The functions of the cdk-cyclin kinase inhibitor p21WAF1, Pathol. Biol. (Paris) 48 (2000) 190–202.
[8] G.P. Dotto, p21(WAF1/Cip1): more than a break to the cell cycle? Biochim. Biophys. Acta 1471 (2000) M43–M56.
[9] H. Kitaura, M. Shinshi, Y. Uchikoshi, T. Ono, S.M. Iguchi-Ariga, H. Ariga, Reciprocal regulation via protein–protein interaction between c-Myc and p21(cip1/waf1/sdi1) in DNA replication and transcription, J. Biol. Chem. 275 (2000) 10477–10483.
[10] L. Delavaine, N.B. La Thangue, Control of E2F activity by p21Waf1/Cip1, Oncogene 18 (1999) 5381–5392.
[11] N.A. Timchenko, T.E. Harris, M. Wilde, T.A. Bilyeu, B.L. Burgess-Beusse, M.J. Finegold, G.J. Darlington, CCAAT/enhancer binding

protein alpha regulates p21 protein and hepatocyte proliferation in newborn mice, Mol. Cell. Biol. 17 (1997) 7353–7361.
[12] O. Coqueret, H. Gascan, Functional interaction of STAT3 transcription factor with the cell cycle inhibitor p21WAF1/CIP1/SDI1, J. Biol. Chem. 275 (2000) 18794–18800.
[13] A. Suzuki, Y. Tsutomi, K. Akahane, T. Araki, M. Miura, Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP, Oncogene 17 (1998) 931–939.
[14] J. Shim, H. Lee, J. Park, H. Kim, E.J. Choi, A non-enzymatic p21 protein inhibitor of stress-activated protein kinases, Nature 381 (1996) 804–806.
[15] M. Asada, T. Yamada, H. Ichijo, D. Delia, K. Miyazono, K. Fukumuro, S. Mizutani, Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation, EMBO J. 18 (1999) 1223–1234.
[16] J.M. Estanyol, M. Jaumot, O. Casanovas, A. Rodriguez-Vilarrupla, N. Agell, O. Bachs, The protein SET regulates the inhibitory effect of p21(Cip1) on cyclin E-cyclin-dependent kinase 2 activity, J. Biol. Chem. 274 (1999) 33161–33165.
[17] M. Taules, A. Rodriguez-Vilarrupla, J.M. Rius, E. Estanyol, O. Casanovas, D.B. Sacks, E. Perez-Paya, O. Bachs, N. Agell, Calmodulin binds to p21(Cip1) and is involved in the regulation of its nuclear localization, J. Biol. Chem. 274 (1999) 24445–24448.
[18] J.O. Funk, S. Waga, J.B. Harry, E. Espling, B. Stillman, D.A. Galloway, Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein, Genes Dev. 11 (1997) 2090–2100.
[19] J.M. Kearsey, P.J. Coates, A.R. Prescott, E. Warbrick, P.A. Hall, Gadd45 is a nuclear cell cycle regulated protein which interacts with p21Cip1, Oncogene 11 (1995) 1675–1683.
[20] T. Ono, H. Kitaura, H. Ugai, T. Murata, K.K. Yokoyama, S.M. Iguchi-Ariga, H. Ariga, TOK-1, a novel p21Cip1-binding protein that cooperatively enhances p21-dependent inhibitory activity toward CDK2 kinase, J. Biol. Chem. 275 (2000) 31145–31154.
[21] C. Gotz, P. Wagner, O.G. Issinger, M. Montenarh, p21WAF1/CIP1 interacts with protein kinase CK2, Oncogene 13 (1996) 391–398.
[22] H.T. Cuypers, G. Selten, W. Quint, M. Zijlstra, E.R. Maandag, W. Boelens, P. van Wezenbeek, C. Melief, A. Berns, Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region, Cell 37 (1984) 141–150.
[23] M. Breuer, R. Slebos, S. Verbeek, M. van Lohuizen, E. Wientjens, A. Berns, Very high frequency of lymphoma induction by a chemical carcinogen in pim-1 transgenic mice, Nature 340 (1989) 61–63.
[24] G. Selten, H.T. Cuypers, W. Boelens, E. Robanus-Maandag, J. Verbeek, J. Domen, C. van Beveren, A. Berns, The primary structure of the putative oncogene pim-1 shows extensive homology with protein kinases, Cell 46 (1986) 603–611.
[25] M. van Lohuizen, S. Verbeek, P. Krimpenfort, J. Domen, C. Saris, T. Radaszkiewicz, A. Berns, Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors, Cell 56 (1989) 673–682.
[26] T. Moroy, S. Verbeek, A. Ma, P. Achacoso, A. Berns, F. Alt, E mu N- and E mu L-myc cooperate with E mu pim-1 to generate lymphoid tumors at high frequency in double-transgenic mice, Oncogene 6 (1991) 1941–1948.
[27] D. Acton, J. Domen, H. Jacobs, M. Vlaar, S. Korsmeyer, A. Berns, Collaboration of pim-1 and bcl-2 in lymphomagenesis, Curr. Top. Microbiol. Immunol. 182 (1992) 293–298.
[28] T. Schmidt, H. Karsunky, E. Gau, B. Zevnik, H.P. Elsasser, T. Moroy, Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis, Oncogene 17 (1998) 2661–2667.
[29] Z. Wang, N. Bhattacharya, M. Weaver, K. Petersen, L. Gapter, N.S. Magnuson, Pim-1: a serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis, J. Vet. Sci. 2 (2001) 167–179.
[30] N. Zhu, L.M. Ramirez, R.L. Lee, N.S. Magnuson, G.A. Bishop, M.R. Gold, CD40 signaling in B cells regulates the expression of the Pim-1 kinase via the NF-kappa B pathway, J. Immunol. 168 (2002) 744–754.
[31] D. Hoover, M. Friedmann, R. Reeves, N.S. Magnuson, Recombinant human pim-1 protein exhibits serine/threonine kinase activity, J. Biol. Chem. 266 (1991) 14018–14023.
[32] C.K. Palaty, I. Clark-Lewis, D. Leung, S.L. Pelech, Phosphorylation site substrate specificity determinants for the Pim-1 protooncogene-encoded protein kinase, Biochem. Cell. Biol. 75 (1997) 153–162.
[33] J.D. Leverson, P.J. Koskinen, F.C. Orrico, E.M. Rainio, K.J. Jalkanen, A.B. Dash, R.N. Eisenman, S.A. Ness, Pim-1 kinase and p100 cooperate to enhance c-Myb activity, Mol. Cell 2 (1998) 417–425.
[34] T. Mochizuki, C. Kitanaka, K. Noguchi, T. Muramatsu, A. Asai, Y. Kuchino, Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway, J. Biol. Chem. 274 (1999) 18659–18666.
[35] N. Koike, H. Maita, T. Taira, H. Ariga, S.M. Iguchi-Ariga, Identification of heterochromatin protein 1 (HP1) as a phosphorylation target by pim-1 kinase and the effect of phosphorylation on the transcriptional repression function of HP1, FEBS Lett. 467 (2000) 17–21.
[36] H. Maita, Y. Harada, D. Nagakubo, H. Kitaura, M. Ikeda, K. Tamai, K. Takahashi, H. Ariga, S.M. Iguchi-Ariga, PAP-1, a novel target protein of phosphorylation by pim-1 kinase, Eur. J. Biochem. 267 (2000) 5168–5178.
[37] Z. Wang, N. Bhattacharya, M.K. Meyer, H. Seimiya, T. Tsuruo, N.S. Magnuson, Pim-1 negatively regulates the activity of ptp-u2s phosphatase and influences terminal differentiation and apoptosis of monoblastoid leukemia cells, Arch. Biochem. Biophys. 390 (2001) 9–18.
[38] E.M. Rainio, J. Sandholm, P.J. Koskinen, Cutting edge: transcriptional activity of NFATc1 is enhanced by the Pim-1 kinase, J. Immunol. 168 (2002) 1524–1527.
[39] Y. Ishibashi, H. Maita, M. Yano, N. Koike, K. Tamai, H. Ariga, S.M. Iguchi-Ariga, Pim-1 translocates sorting nexin 6/TRAF4-associated factor 2 from cytoplasm to nucleus, FEBS Lett. 506 (2001) 33–38.
[40] N. Bhattacharya, Z. Wang, C. Davitt, I.F. McKenzie, P.X. Xing, N.S. Magnuson, Pim-1 associates with protein complexes necessary for mitosis, Chromosoma 111 (2002) 80–95.
[41] W. Wei, R.M. Hemmer, J.M. Sedivy, Role of p14(ARF) in replicative and induced senescence of human fibroblasts, Mol. Cell. Biol. 21 (2001) 6748–6757.
[42] M. Friedmann, M.S. Nissen, D.S. Hoover, R. Reeves, N.S. Magnuson, Characterization of the proto-oncogene pim-1: kinase activity and substrate recognition sequence, Arch. Biochem. Biophys. 298 (1992) 594–601.
[43] M.I. Olnes, R.N. Kurl, Isolation of nuclear extracts from fragile cells: a simplified procedure applied to thymocytes, Biotechniques 17 (1994) 828–829.
[44] J.A. Cooper, B.M. Sefton, T. Hunter, Detection and quantification of phosphotyrosine in proteins, Methods Enzymol. 99 (1983) 387–402.
[45] W.J. Boyle, G.P. van der Geer, T. Hunter, Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates, Methods Enzymol. 201 (1991) 110–149.
[46] M.T. Scott, N. Morrice, K.L. Ball, Reversible phosphorylation at the C-terminal regulatory domain of p21(Waf1/Cip1) modulates proliferating cell nuclear antigen binding, J. Biol. Chem. 275 (2000) 11529–11537.
[47] B.P. Zhou, Y. Liao, W. Xia, B. Spohn, M.H. Lee, M.C. Hung, Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells, Nat. Cell Biol. 3 (2001) 245–252.
[48] D. Rousseau, D. Cannella, J. Boulaire, P. Fitzgerald, A. Fotedar, R. Fotedar, Growth inhibition by CDK-cyclin and PCNA binding domains of p21 occurs by distinct mechanisms and is regulated by ubiquitin–proteasome pathway, Oncogene 18 (1999) 4313–4325.

[49] K. Sasaki, K. Ikeda, M. Nagai, H. Shima, M. Nagao, J. Takahara, S. Irino, Protein phosphatase 2A alpha involvement in granulocytic differentiation of HL-60 cells, Hematol. Oncol. 11 (1993) 1–5.
[50] S.B. Omay, H. Ogasawara, H. Toyoda, K. Nakai, H. Shima, M. Nagao, M.C. Mumby, H. Shiku, M. Nishikawa, Translocation of protein phosphatase 1 catalytic subunits during 1,25-dihydroxyvitamin D3-induced monocytic differentiation of HL-60 cells, Cancer Res. 55 (1995) 774–780.
[51] N.E. Faulkner, J.M. Hilfinger, D.M. Markovitz, Protein phosphatase 2A activates the HIV-2 promoter through enhancer elements that include the pets site, J. Biol. Chem. 276 (2001) 25804–25812.
[52] J.A. Erhardt, R.N. Pittman, Ectopic p21(WAF1) expression induces differentiation-specific cell cycle changes in PC12 cells characteristic of nerve growth factor treatment, J. Biol. Chem. 273 (1998) 23517–23523.
[53] C. Cayrol, B. Ducommun, Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21, Oncogene 17 (1998) 2437–2444.
[54] A. Suzuki, H. Kawano, M. Hayashida, Y. Hayasaki, Y. Tsutomi, K. Akahane, Procaspase 3/p21 complex formation to resist fas-mediated cell death is initiated as a result of the phosphorylation of p21 by protein kinase A, Cell Death Differ. 7 (2000) 721–728.
[55] R.W. Kriwacki, L. Hengst, L. Tennant, S.I. Reed, P.E. Wright, Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11504–11509.
[56] A. Toker, Signaling through protein kinase C, Front. Biosci. 3 (1998) D1134–D1147.
[57] A. Feliciello, M.E. Gottesman, E.V. Avvedimento, The biological functions of A-kinase anchor proteins, J. Mol. Biol. 308 (2001) 99–114.
[58] D.R. Alessi, M. Deak, A. Casamayor, F.B. Caudwell, N. Morrice, D.G. Norman, P. Gaffney, C.B. Reese, C.N. MacDougall, D. Harbison, A. Ashworth, M. Bownes, 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase, Curr. Biol. 7 (1997) 776–789.
[59] D. Stokoe, L.R. Stephens, T. Copeland, P.R. Gaffney, C.B. Reese, G.F. Painter, A.B. Holmes, F. McCormick, P.T. Hawkins, Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B, Science 277 (1997) 567–570.
[60] N. Filippa, C.L. Sable, B.A. Hemmings, E. Van Obberghen, Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation, Mol. Cell. Biol. 20 (2000) 5712–5721.
[61] K. Nishikawa, A. Toker, F.J. Johannes, Z. Songyang, L.C. Cantley, Determination of the specific substrate sequence motifs of protein kinase C isozymes, J. Biol. Chem. 272 (1997) 952–960.
[62] D.R. Alessi, F.B. Caudwell, M. Andjelkovic, B.A. Hemmings, P. Cohen, Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase, FEBS Lett. 399 (1996) 333–338.
[63] K.S. Walker, M. Deak, A. Paterson, K. Hudson, P. Cohen, D.R. Alessi, Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha, Biochem. J. 331 (Pt 1) (1998) 299–308.